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(54) Device and method for providing confined reaction and detection.

(57) It is known to carry out PCR amplification and detection using a reaction vessel in which solid particles are fixed and liquid sample and reagents are moved past these particles. Described herein is a device (10, 30) and method for carrying out such reactions. Solids (for example, beads (16)) are used to obtain separation between bound and "free" label reagents, and these are transferred from a first chamber (12) to a second chamber (32), specifically through a wash liquid so as to wash off the "free" label reagent from the solids (16). The first chamber (12) has a cover (14) which is pierced by prong (40) mounted on cover portion (34) of the second chamber (32) to create passageways (36) for the transfer of the solids (16) from the first chamber (12) to the second chamber (32). The passageways (36) remain contained within the device (10, 30) confining the reaction and detection products therein.

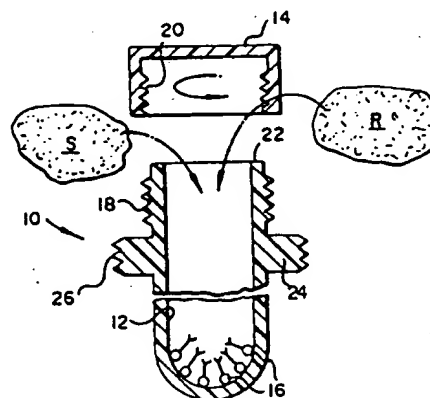


FIG. 1

EP 0 573 098 A2

This invention relates to devices and methods for providing confined reaction and detection such as for PCR amplification and detection.

It is known to carry out polymerase chain reaction (PCR) amplification and detection using a reaction vessel in which solid particles are fixed and liquid sample and reagents are moved sequentially past the fixed solid particles. Such reaction vessels are described, for example, in EP-A-0 381 501. Although such vessels work well to confine amplified DNA from leaking out of the vessels and contaminating other test vessels, they do have a slight disadvantage - the liquid solutions must be carefully moved from place to place, without dislodging the fixed solid particles used for detection. As a result, the reaction vessel is carefully constructed in a way which excludes less expensive, simpler constructions. In particular, liquid flow and attachment of the "fixed" solids must be maintained so that such solids do not migrate away from their read location.

Similarly, immunoassays are known in which a label reagent attached to a target bound to a solid (and called "bound" reagents) are separated from label reagents associated but unattached with the target (and called "free" reagents). This is done by fixing the solids in the test device and washing off the free reagent by flowing a wash liquid past or around the fixed solids. Typically a medium such as a solid filter is used to separate the bound reagents from the free reagents after the wash step. Examples are described in US-A-4 921 677, which work well for their purpose. However, care is needed to select a filtering medium that, while passing free liquid, will not pass the solids bearing the bound reagent.

Thus, in both the PCR amplification and detection, and in the immunoassays noted above, the technique has been to fix or immobilize the solid particles, and to move the liquid.

It was also known in the prior art to pour particulate solids bearing a target, such as for immunoassay, into a detection solution where unbound label reagent is washed off the solids as a bound-free separation. Examples of this can be found in US-A-4 743 538. However, invariably when such solids are poured or flowed into the detection solution, the chamber holding the solids is uncovered to allow the fluid flow. Such uncovering is totally unacceptable as a detection scheme for PCR since the containment needed in PCR amplification becomes lost. Because bulk flow of solids necessitates the opening of sizable channels between the respective compartments, the mere act of providing the bulk flow has in the past been counterproductive to maintaining confinement of PCR reaction products.

It has been a problem, therefore, prior to this invention, to provide a detection vessel and method, suitable for PCR amplification and detection while confined, or for immunoassay, which are less difficult to provide and/or less expensive than the currently available vessels and methods.

More specifically, the problem has been to provide a device and a method of flowing bulk solids between confined locations, namely from an amplifying location to a detecting location, without opening up the amplifying location to the atmosphere so as to cause contamination.

It is therefore an object of the present invention to provide a device and method which overcome the above-noted problems.

More specifically, in accordance with one aspect of the present invention, there is provided a device for reacting a solid with a sample target bearing a label reagent while contained therein, the device comprising:-

- a) a first confined region for attaching the sample target to particulate solids;
- b) at least a second confined region containing at least a wash liquid for washing unbound label reagent from some of the solids;
- c) passage means including at least one passageway between the first confined location and the second confined location to allow communication of liquid between the first confined location and the second confined location; and
- d) confining means for confining and containing the target sample totally within the device when the passage means are opened;

characterized in that some solids are mobile within the device and the passage means are constructed with a size and distribution sufficient to allow the solids to migrate between the first confined location and the second confined location into contact with the liquid label reagent and the wash liquid while keeping the target sample totally confined and contained within the device.

In accordance with another aspect of the present invention, there is provided a process for reacting and detecting particulate solids with a sample target bearing a label reagent while contained in a device according to any one of the preceding claims, the process comprising:-

- a) providing sufficient replicas of the target as to be detectable;
- b) interacting the detectable target with some particulate solids; and
- c) detecting label reagent on the target and the solids, all while being confined within the device during and after step a).

characterized in that the particulate solids are mobile and the process further includes the step of pouring particulate solids reacted with sample target bearing a label reagent, into a wash liquid to

remove unbound label reagent.

Accordingly, it is an advantageous feature of the invention that no care is needed, in a contained reaction and containment device, in preventing detectable solids from being displaced from a read location, since it is the solids which are mobile and moved, to a fixed liquid, rather than vice-versa.

It is a related advantageous feature that solids bearing both bound and free reagents can be poured into a wash solution for bound/free separation while being retained in a closed, contained environment preventing contamination.

It is a further advantageous feature of the invention that a device and method are provided which avoid processing difficulties inherent in devices that fix solid particles and move liquids relative thereto.

For a better understanding of the present invention, reference will now be made, by way of example only, to the accompanying drawings in which:-

Figure 1 is an exploded, partially schematic sectioned elevational view of the first confined region of a first embodiment of a device in accordance with the present invention;

Figure 2 is a view similar to that shown in Figure 1 but also illustrating the second confined region, in its cooperation with the first confined region, and also the means for confining the target during the processing of the present invention;

Figure 3 is a view similar to that shown in Figure 2 but illustrating a second embodiment of the present invention prior to providing a passageway through the confinements of the various confined regions;

Figure 4 is a fragmentary sectioned elevational view of only the lower half of the device, following the opening of passageways in the confinements shown in Figure 3;

Figure 5 is a view similar to that shown in the lower half of Figure 3, but illustrating a third embodiment of the present invention; and

Figures 6 and 7 are each a plot or graph depicting assay sensitivity.

The invention is hereinafter described particularly in connection with certain preferred embodiments, in which PCR amplification and detection takes place within a contained environment to prevent contamination, such as one which is tubular in shape. In addition, it is applicable regardless of whether the reaction scheme is PCR amplification or some other reaction such as an immunoassay, as long as a target sample (for example, an antibody or antigen), is provided in sufficient quantity as to be detectable, is interacted with both particulate solids and at least a liquid label reagent, in a manner which requires the solids to be mobile so

as to move into the label reagent. Also, it is applicable regardless of the shape of the confined locations or the overall device.

As used herein, a "sample target bearing a label reagent" is preferably a replicated oligonucleotide sequence ending in either a label reagent per se, or in biotin which subsequently reacts with an avidin-bearing label reagent. If the sequence ends in a label reagent per se, that label reagent is preferably a fluorescing dye capable of resisting repeated temperature increases of up to 95°C without losing its ability to fluoresce at a detectable level. Examples of such dyes include fluoresceins or coumarins.

Additionally, "sample target bearing a label reagent" can be an antigen (or antibody) which is complexed with an antibody (or antigen) bearing a label reagent per se, or biotin which subsequently reacts with an avidin-bearing label reagent.

As used herein "label reagent" is a reagent, preferably in solution, capable of directly or indirectly revealing the presence of the sample target. A label reagent is "directly revealing" if it can be detected by stimulating it with outside energy, for example, if it is a fluorescing moiety which fluoresces when exposed to light. It is "indirectly revealing" if it requires a "detector reagent" also in the device, for example, if it includes an enzyme which requires a substrate also in the device, to produce a color for example.

Also as used herein, "interacting the target with both particulate solids and at least a liquid label reagent" means either sequentially as usually occurs at two separate reactive sites on the sample target, for example, an antigen with two epitope sites each reactive sequentially with an antibody on a solid, and then with a labeled antibody; or simultaneously such as occurs when a target antigen interacts with a soluble labeled antigen competitively for an antibody on a solid.

Particularly useful label reagents comprise enzymes attached to strep-avidin, such as peroxidases and particularly horse-radish peroxidase. When the latter enzyme is used, a useful substrate is H₂O₂ and a triarylimidazole dye such as those described in US-A-4 870 388 and US-A-4 089 747.

"Particulate solids" as used herein refer to any solids to which the target can be readily bound, preferably those which are relatively small particulates, for example, those having as their maximum dimension, a value no greater than 300µm. Highly preferred are beads of polystyrene dimensioned to be 0.1-100µm.

Referring now to the features of the invention in combination, each of the features a) to d) of the device described above is already known by itself. In particular, they appear generically in EP-A-0 381 501, wherein however the particulate solids are

fixed and the liquid reagents flow over those solids.

Thus, a device constructed in accordance with the invention includes, Figure 1, a first confined region 10 provided by a chamber 12 with a removable sealing cap 14, which is applied to seal the device after all the reagents R and sample S needed for PCR amplification are in place. Pre-included in chamber 12 are beads 16 bearing a linking group shown as a "Y", which group is preferably an oligonucleotide probe designed to anneal to the target DNA which is to be amplified in chamber 12. Both the sample S and all the needed reagents for amplification are added to the device and cap 14 is sealed. Any sealing means can be used, but preferably they comprise a male and female thread 18, 20, with preferably the male thread being on the top portion 22 of chamber 12.

Chamber 12 has on its outside, confining means for allowing it to securely attach to the remaining portion(s) 30 of the device, in a manner which allows the contents of chamber 12 to be poured into portion 30 while keeping all liquids confined within the device. Preferably the confining means comprise a protruding lip 24 on which is mounted male screw threads 26.

Reagents R preferably also include a primer for the targeted nucleic acid sequence to which is attached a fluorescing label.

Thereafter, PCR amplification occurs in chamber 12 using known temperature cycling, to provide sufficient labeled replicas of the targeted nucleic acid sequence, such as DNA, as can be readily detected in other portions of the device and method. To ensure the amplification is not unduly hindered by premature annealing to the beads, the probe on the bead is selected to have a lower T_m , that is, a lower melt temperature than the T_m of the amplification primers, as is well-known.

The result is that some beads 16 have, Figure 2, targeted nucleic acid sequence bound to them, as shown by the "Xs".

At this point, beads 16 of chamber 12 also bear on them, loosely attached, unannealed primers bearing the fluorescing medium. Some of the beads may have only such loosely attached, unannealed primers.

Turning next to Figure 2, the other portion of the device comprises a second confined region 30, including a chamber 32 having a bottom wall 33, covered by cover 34 apertured at 36. Center portion 38 of cover 34 has a piercing prong 40 capable of bursting through cover 14 on contact. Rising above cover 34 is a sleeve 42 that is provided with female threads 44 for engagement with threads 26 of chamber 12. When threads 44 and 26 are initially engaged, cover 14 is spaced away from contact with prong 40, as shown by the raised phantom 14' position of the cover. However, as

chamber 12 is further screwed into sleeve 42, prong 40 penetrates cover 14 sufficiently to provide a passageway for solids 16 and any reagents thereon.

The liquid content of chamber 32 is at least a wash liquid. The effect of the wash liquid is to wash off the beads, as they fall by reason of force "F" to the bottom wall 33, any loosely "attached" unannealed labeled primer. Such washed-off primer stays near the surface "S" of the wash liquid. Force "F" can be supplied either as gravity or as a centrifugal force.

What then reaches bottom wall 33 is only the beads and label reagent which is part of the targeted DNA due to the amplification process (as annealed to the beads). The label reagent, by reason of its fluorescing capability, can then be detected by emitting light of the appropriate wavelengths, at the bottom region of chamber 32, in the direction indicated by arrow 50.

Thus, amplification, wash and detection all occur within a sealed, contained device, even when the transfer mechanism is one of transfer of solids from first region 10 to second region 30, and not just liquids.

As noted above, the label reagent need not be a fluorescing moiety, but can instead comprise an enzyme which cooperates with a substrate to produce a detectable color. In that case, a three-chambered device is preferred, Figs 3 and 4. Parts similar to those previously described bear the same reference numeral, to which the distinguishing suffix "A" is appended.

Thus, the device comprises two cooperating confining portions 10A and 30A, screw-attached by mating threads 26A and 44A to allow piercing of cover 14A as before. (Cover 14A can be screw-attached as in the previous embodiment.) However, an additional confined region 70 is added by extending the walls of chamber 32A and inserting region 70 above a barrier means 74, to create a third chamber 72 located between barrier means 74 and cover 34A.

In this construction, the liquid within chamber 72 comprises an enzyme chemically modified to react with targeted nucleic acid sequences produced in chamber 12A. (If immunoassays are involved, the chemical modification of the enzyme allows it to react with an antibody complexed to the targeted antigen.) Preferred is the use of avidin chemically reacted with the enzyme, in which case the targeted nucleic acid sequences end in biotin. A highly preferred example of the liquid in region 70 is strep-avidin horseradish peroxidase.

If the enzyme is a peroxidase, then H_2O_2 is also included in chamber 72 in portion 30A.

A variety of barrier means 74 are useful. In the embodiment of Figures 3 and 4, barrier means 74

preferably are liquifiable under the influence of either a temperature change or of centrifugal force. Examples of the former include a wax which liquifies when heated to a temperature less than that which deactivates the enzyme, for example, a temperature less than 60°C. Paraffin is a preferred example.

Examples of a barrier means which liquifies under centrifugal force include thixotropic gels which will allow passage of the beads at a high centrifugal force "CF", for example, a force of at least 10,000g. A preferred example of such a gel includes the gel available in tubes sold by Sarstedt Corp; under the tradename "Gel Monovette", usually comprising silica and a polyester.

In this embodiment, the liquid content of confined region 30A comprises a substrate for the enzyme of chamber 72, for example, a leuco dye in aqueous solution. Thus, the liquid content provides two functions: it provides the washing of the beads as they move past barrier means 74, Figure 4, to remove loosely "attached" enzyme which is not reacted by the avidin-biotin linkage to the beads, and it provides the substrate for the enzyme.

The beads which collect on bottom wall 33A are then detected by their color change. Any color change created at the top portion 80 of chamber 30A due to the washed-off loose enzyme, can be readily spatially distinguished from the color change of the beads at wall 33A.

It is not essential that barrier means 74 be liquifiable, or indeed, be anything other than a rigid structure. Thus, Figure 5, it can be a wall structure cutting off chamber 72 from everything below, including chamber 32A. Parts similar to those previously described bear the same reference numeral to which the distinguishing suffix "B" has been appended.

As shown in Figure 5, the device is identical to those previously described, insofar as middle region 70B, its chamber 72B, and the first confined region (not shown) are concerned, except that barrier means 74B now comprises a thin wall which is an extension of the walls of chamber 72B, so as to close off access out of that chamber. In addition, a skirt 90 extends down below barrier means 74B, which is internally threaded at 92. Third confined region 30B then comprises a chamber 32B with a bottom wall 33B as before, except that at the top 94 of region 30B, there are disposed an apertured cover 34B constructed substantially identically as cover 34A, and external threads 96 that mate with threads 92. Thus, prong 40B acts to break through barrier 74B simply by screwing chamber 32B farther into skirt 90. In either position, the threaded engagement of skirt 90 by threads 96 ensures that no leakage can occur out of the device. When

prong 40B does burst through barrier wall 74B, the solids (beads) in chamber 72B then flow down into chamber 32B as before, to wash off unbound label reagent and to react bound label reagent to produce colored beads at the bottom, adjacent to wall 33B.

The following non-limiting examples further illustrate the invention:

Example 1

To show that the barrier means and washing step function as described above, synthetic DNA samples were prepared at concentrations from 200 pmoles down to 0 pmoles. The DNA was suspended in a tris(hydroxymethyl)-aminomethane-ethylenediaminetetraacetic acid buffer, then diluted 1:30 in a high salt buffer.

DNA probes were covalently attached to beads of poly(styrene-co-3-(p-vinylbenzylthio)propionic acid) (weight ratio 95:5) (approximately 1 micron). The beads were suspended at 0.24% solids and 6% Celite™ diatomaceous earth in a streptavidin-HRP (SA-HRP) solution.

Microcentrifuge tubes were prepared containing a bottom layer of 100μl 0.5% agarose/4% Sodium chloride in 4,5-bis(4-dimethylaminophenyl)-2-(4-hydroxy-3,5-dimethoxyphenyl)imidazole leuco dye solution. A second layer contained 500μl of 0.5% agarose/2% sodium chloride in water.

200μl of a DNA sample was denatured at 95°C for 5min. 100μl of the bead/Celite/SA-HRP was added to the sample. The sample was vortexed for 5s, then 100μl of the sample was added to the prepared microcentrifuge tube to simulate the embodiment of Figure 3. (No cover 14 or 14A was used.)

The tube was centrifuged for 5min at 14,000rpm, then the color of the pellet was observed.

As seen in the graph of Figure 6 this method detects as low as 0.05nmoles/l.

Example 2

The procedure of Example 1 was repeated, except that the suspension of the beads was at 1.2% solids, and the diatomaceous earth was replaced with 1% Zonyl FSN, a non-ionic, fluorinated surfactant available from duPont de Nemours. The tubes were used in Sarstedt "microvette SCB 1000" gel tubes, and agarose was left out of the leuco dye solution. The sole barrier means (74 in Figure 3) was the Sarstedt gel. The concentration of NaCl was boosted to 15%. The processing occurred as follows: the tubes so prepared were centrifuged for 1min at 14,000rpm and rinsed in deionized and distilled, microfiltered water, only for

the purpose of locating the leuco dye solution below the barrier means. Then 50µl of a Streptavidin-HRP (SA-HRP) solution with 5% Celite was layered on the gel.

307µl of a DNA sample was denatured at 95°C for 5min. 34µl of the bead/Zonyl FSN was added to the sample. The sample incubated at 42°C for 5min. Then 100µl of the sample was added to the prepared tube.

The tube was centrifuged 5min at 14,000rpm, then the color of the pellet was observed.

As seen in the graph of Figure 7, this method detects as low as 0.3nmoles/l sample and no color is observed when no sample is present.

The invention disclosed herein may be practiced in the absence of any element which is not specifically disclosed herein.

Claims

1. A device (10, 30; 10A, 30A, 70; 30B, 70B) for reacting a solid with a sample target (S) bearing a label reagent (R) while contained therein, the device comprising:-

- a) a first confined region (10, 12, 14; 10A, 12A, 14A) for attaching the sample target (S) to particulate solids (16);

- b) at least a second confined region (30, 32, 33, 34; 30A, 32A, 33A, 34A; 30B, 32B, 33B, 34B) containing at least a wash liquid for washing unbound label reagent (R) from some of the solids (16);

- c) passage means (36, 40; 40B) including at least one passageway (36) between the first confined location (10, 12, 14; 10A, 12A, 14A) and the second confined location (30, 32, 33, 34; 30A, 32A, 33A, 34A; 30B, 32B, 33B, 34B) to allow communication of liquid between the first confined location (10, 12, 14; 10A, 12A, 14A) and the second confined location (30, 32, 33, 34; 30A, 32A, 33A, 34A; 30B, 32B, 33B, 34B); and

- d) confining means (18, 20, 26, 44; 26A, 44A) for confining and containing the target sample (S) totally within the device (10, 30; 10A, 30A, 70; 30B, 70B) when the passage means (36, 40; 40B) are opened;

characterized in that some solids (16) are mobile within the device (10, 30; 10A, 30A, 70; 30B, 70B) and the passage means (36) are constructed with a size and distribution sufficient to allow the solids (16) to migrate between the first confined location (10, 12, 14; 10A, 12A, 14A) and the second confined location (30, 32, 33, 34; 30A, 32A, 33A, 34A; 30B, 32B, 33B, 34B) into contact with the liquid label reagent and the wash liquid while keeping the target sample totally confined and con-

tained within the device (10, 30; 10A, 30A, 70; 30B, 70B).

2. A device according to claim 1, wherein the first confined location (10, 12, 14; 10A, 12A, 14A) comprises a first chamber (12; 12A) and a temporary cover (14; 14A) therefor capable of completely sealing the first chamber (12; 12A), the second confined location (30, 32, 33, 34; 30A, 32A, 33A, 34A; 30B, 32B, 33B, 34B) comprises a second chamber (32; 32A; 32B) and an apertured cover (34; 34A; 34B) therefor, the passage means (36, 40; 40B) comprising piercing means (40; 40B) for piercing the temporary cover (34; 34A; 34B) on contact, and the confining means (18, 20, 26, 44; 26A, 44A) comprises threaded means (18, 20, 26, 44; 26A, 44A) for removably holding together the first chamber (12; 12A) and the second chamber (32; 32A) in two spaced-apart positions, one position being effective to keep the piercing means (40; 40B) out of contact with the temporary cover (34; 34A; 34B) and the other position being effective to pierce the temporary cover (34; 34A; 34B) with piercing means (40; 40B).

3. A device according to claim 1 or 2, further including a third confined location (70, 72, 74; 70B, 72B, 74B) located between the first confined location (10A, 12A, 14A) and the second confined location (30A, 32A, 33A, 34A; 30B, 32B, 33B, 34B) and containing a liquid.

4. A device according to claim 3, wherein the liquid in the third confined location (70, 72, 74; 70B, 72B, 74B) comprises a liquid label reagent capable of reacting with a moiety on the sample target (S) to provide a bound complex not removable from the solids (16) by the wash liquid.

5. A device according to claim 3, wherein the liquid in the second confined location (30A, 32A, 33A, 34A; 30B, 32B, 33B, 34B) comprises a detector reagent reactive with the label reagent to directly reveal the presence of the target, the third confined location (70, 72, 74; 70B, 72B, 74B) including separating means (74; 74B) for temporarily separating the second confined location (30A, 32A, 33A, 34A; 30B, 32B, 33B, 34B) and third confined location (70, 72, 74; 70B, 72B, 74B) from liquid communication, the separating means (74; 74B) being capable of change to create a passageway therethrough to provide flow of solid beads (16) between the second confined location (30A, 32A, 33A, 34A; 30B, 32B, 33B, 34B) and

- third confined location (70, 72, 74; 70B, 72B, 74B), the confining means (26A, 44A) being effective to contain target sample within the device (10A, 30A, 70; 30B, 70B) when a passageway is created within the separating means (74; 74B).
6. A device according to any one of claims 3 to 5, wherein the wash is located within the second confined location (30A, 32A, 33A, 34A; 30B, 32B, 33B, 34B).
7. A device according to claim 6, wherein the wash and the detector reagent comprise a single solution.
8. A device according to any one of claims 3 to 7, wherein the third confined location (70B, 72B, 74B) includes a pierceable wall surface (74B) effective to confine all substances within the third confined location (70B, 72B, 74B) until pierced, the second confined location comprising a second chamber (32B) with an apertured cover (34B) and piercing means (40B) for piercing the pierceable wall surface (74B), the device (30B, 70B) further including threaded means (92, 96) for removably holding together the second chamber (32B) and the third chamber (72B) in two spaced-apart positions, one position being effective to keep the piercing means (40B) out of contact with the wall surface (74B) and the other position being effective to pierce the wall surface (74B).
9. A device according to any one of claims 3 to 7, wherein the third confined location (70, 72, 74) comprises a barrier layer (74) of a material which liquifies under either a temperature change or a centrifugal force.
10. A device according to claim 9, wherein the barrier layer (74) comprises a wax which liquifies when heated and wherein the label reagent is thermally stable to at least the temperature of liquification of the barrier layer (74).
11. A device according to any one of the preceding claims, wherein the label reagent (R) comprises an enzyme.
12. A device according to claim 11, wherein the enzyme is a peroxidase.
13. A device according to claim 9, wherein the barrier layer (74) comprises a thixotropic gel effective to pass solid particles when a centrifugal force of at least 10,000g is applied.
14. A process for reacting and detecting particulate solids (16) with a sample target (S) bearing a label reagent (R) while contained in a device (10, 30; 10A, 30A, 70; 30B, 70B) according to any one of the preceding claims, the process comprising:-
- providing sufficient replicas of the target (S) as to be detectable;
 - interacting the detectable target (S) with some particulate solids (16); and
 - detecting label reagent (R) on the target and the solids (16), all while being confined within the device (10, 30; 10A, 30A, 70; 30B, 70B) during and after step a),
- characterized in that the particulate solids (16) are mobile and the process further includes the step of pouring particulate solids (16) reacted with sample target bearing a label reagent, into a wash liquid to remove unbound label reagent.
15. A method according to claim 14, further including the steps of:-
- temporarily separating with separation means, solids reacted with the target during step a) from the liquid label reagent, and
 - then penetrating the separation means (74, 74B) with the solids reacted with the target to allow flow of the solids into the label reagent.
16. A method according to claim 15, further including the step of reacting the label reagent with a detector reagent after penetrating the separation means with the reacted solids, the detector reagent being effective to directly reveal the presence of the target.

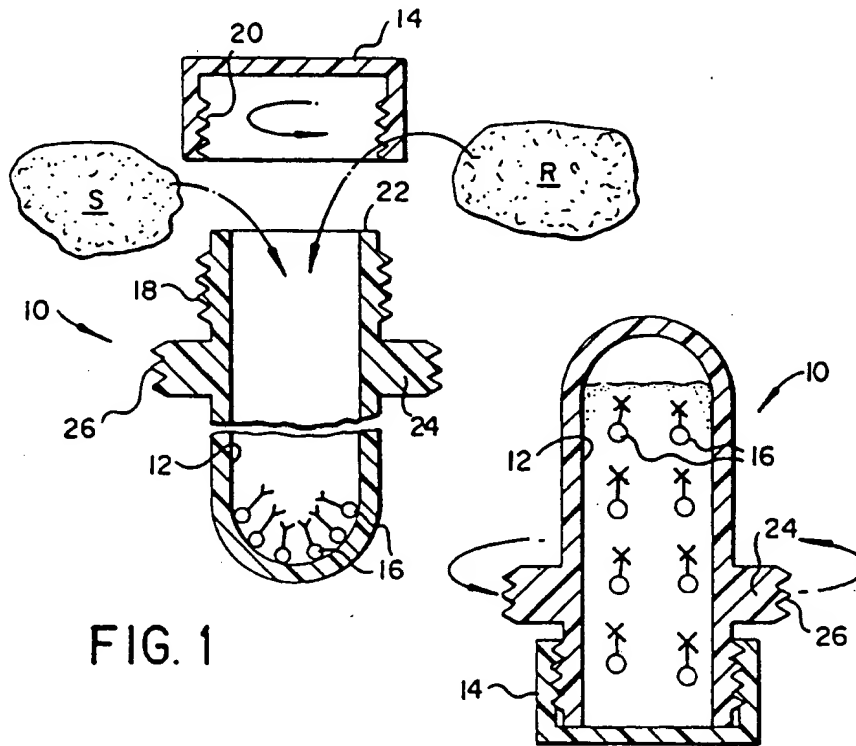


FIG. 1

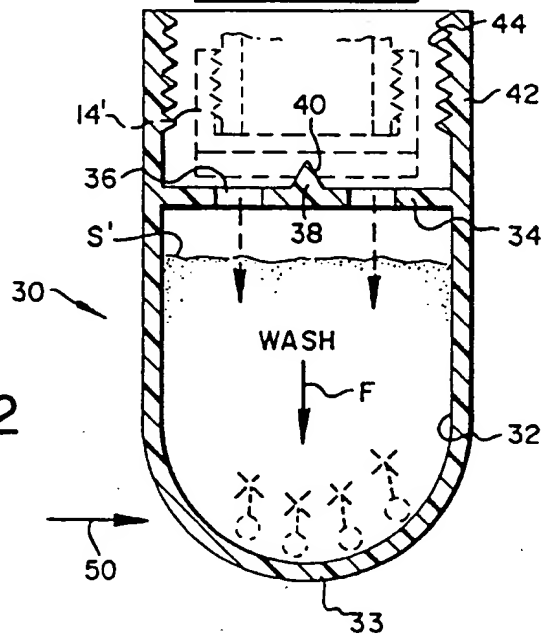


FIG. 2

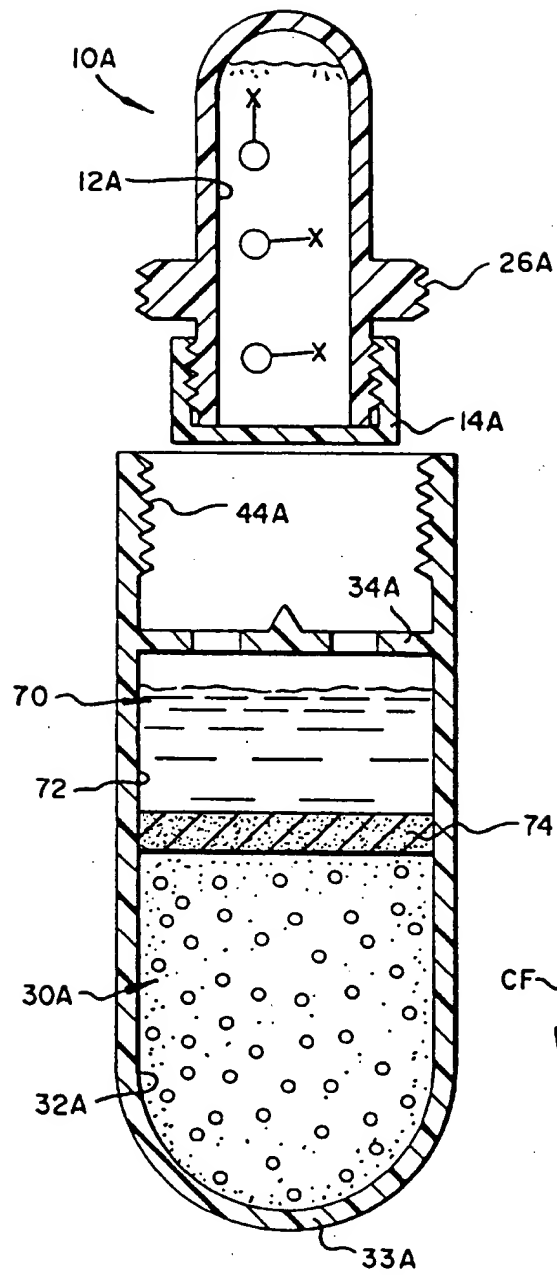


FIG. 3

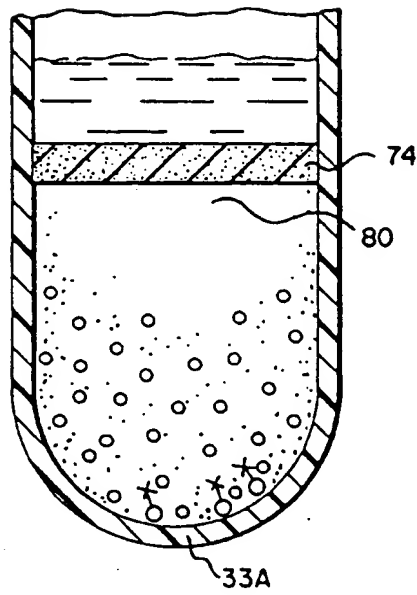


FIG. 4

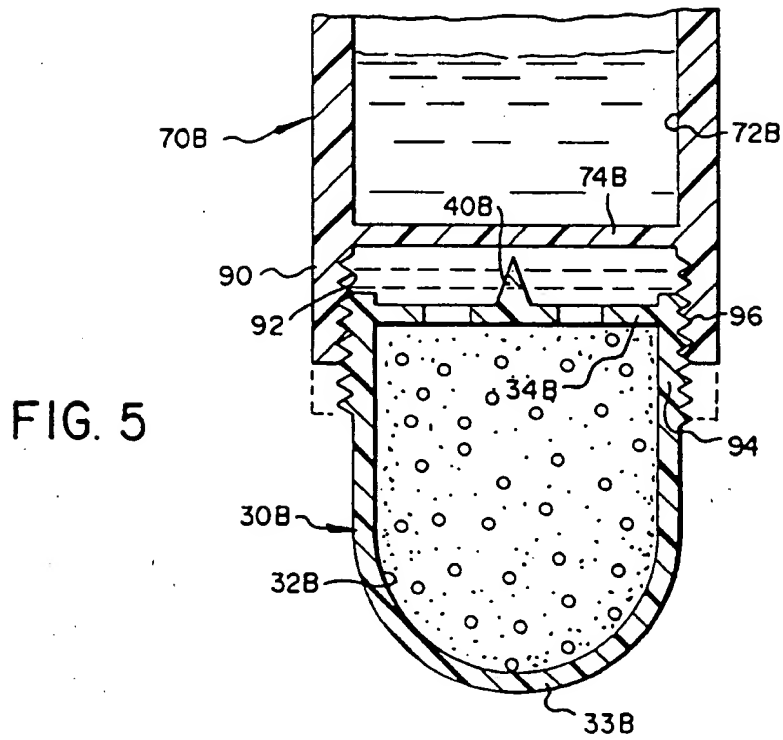


FIG. 5

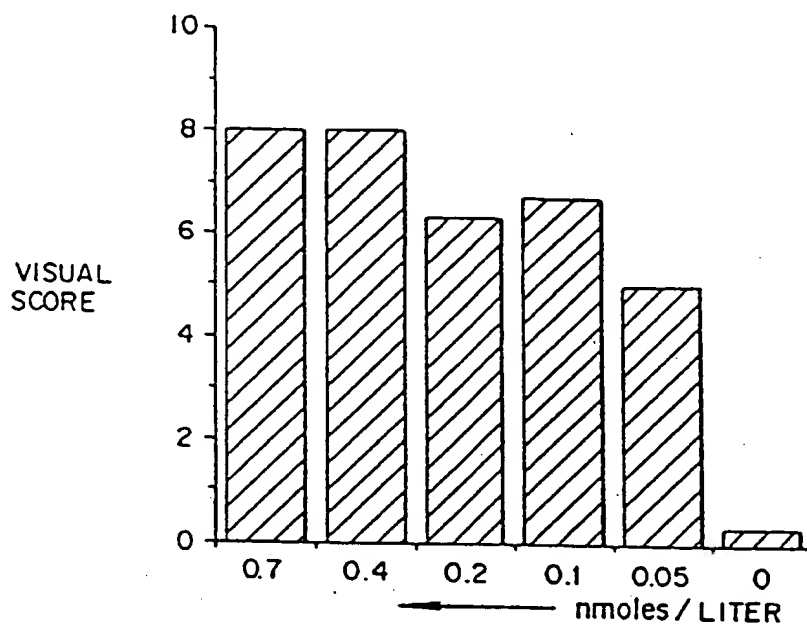


FIG. 6

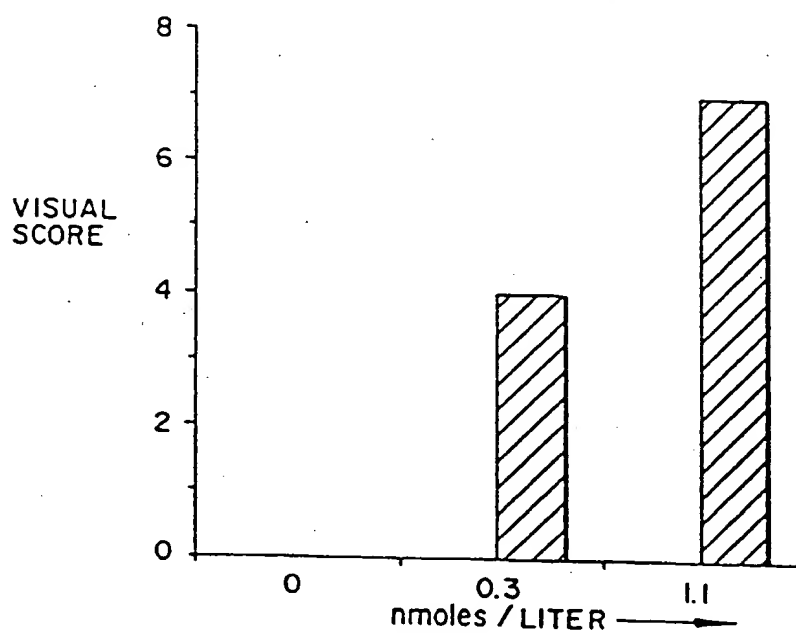


FIG. 7